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For: SURFACE AND ITS MANUFACTURE AND
USES

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Dear Sir:

Applicant hereby claims priority under 35 U.S.C. 119 based on the following prior foreign application filed in the following foreign country on the date indicated:

<u>Country</u>	<u>Application No.</u>	<u>Date</u>
SWEDEN	9901100-9	03/24/1999

In support of this claim, a certified copy of the said original foreign application is filed herewith.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 06-2375, under Order No. 1010831 from which the undersigned is authorized to draw.

Dated: March 12, 2003

Respectfully submitted,

Melissa W. Acosta

By

Melissa W. Acosta

Registration No.: 45,872

FULBRIGHT & JAWORSKI L.L.P.

1301 McKinney, Suite 5100

Houston, Texas 77010-3095

(713) 651-5151

(713) 651-5246 (Fax)



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Christina Vängborg
Christina Vängborg

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SURFACE AND ITS MANUFACTURE AND USES

The present invention concerns a method for enhancing the hydrophilicity of a polymer surface by treatment with a plasma.

A common method for surface modification of plastics is to subject them to various forms of plasma treatment (Chan et al., Surface Science Reports 24 (1996) 1-54; and Garbassi et al, Polymer Surfaces - From Physics to Technology, John Wiley (1998) 238-241). This is done in a plasma reactor, which is a vacuum vessel with a gas at low pressure (typically 10 to 1000 mTorr). When a high frequency electric field is applied over the reactor, a plasma (also called glow discharge) is formed, containing reactive species like ions, free radicals and vacuum-UV photons. These species react with the plastics surface and cause a chemical modification with properties depending on the nature of the gas and on the plasma parameters. Gases like oxygen and argon are typically used for hydrophilisations and adhesion improvement on nonpolar plastics, while vapours of polymerising monomers can be used to apply thin coatings on plastics for a number of different purposes (Yasuda, Plasma Polymerization, Academic Press 1985).

There are a number of publications on treatment of polycarbonate surfaces with oxygen and argon plasmas. A stability study (Morra et al, Angew. Makromol. Chem. 189(3184) (1991) 125-136) showed that much of the hydrophilicity of the treated surfaces was lost after either water extraction or 3 days' dry storage. The hydrophilicity loss after water extraction was due to the formation of low molecular weight water-soluble surface species during the plasma treatment and the storage instability was attributed to rearrangement of the polymer chains in the surface. An ESCA study in reference (Greenwood et al., Macromolecules 30 (1997) 1091-1098) showed that 79% of the oxygen incorporated in the polycarbonate surface by oxygen plasma treatment was removed by washing with a 1:1 cyclohexane/isopropanol mixture. This is again attributed to degradation of polymer chains during the plasma treatment.

Similar effects have also been observed for polystyrene. An ESCA-study of a plasma-treated tissue-culture polystyrene showed about 35% loss of surface oxygen after water washing (Onyiriuka et al: J Coll Interf Sci 144(1), 98 (1991)). In two

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other ESCA studies, oxygen-plasma treated polystyrene gave 25% surface oxygen loss after water washing (Callen et al: J Vac Sci Technol A 13(4), 2023-2029 (1995)), (Morra et al: Angew Macromol Chem 189(3184), 125-136 (1991)). A polystyrene surface 5 treated with an oxygen plasma had initially a water-contact angle of 7°, but after a methanol wash the contact angle increased to 64° (Murakami et al: J Coll Interf Sci 202, 37-44 (1998)).

The electric excitation field typically has a frequency in 10 the radiowave or microwave region, i.e. kHz-MHz or GHz respectively. The modification (hydrophilisation) on the polymer surface caused by the plasma will depend mainly on a number of internal plasma parameters such as: type of species present in the plasma, spatial distributions, energy 15 distributions and directional distributions. In turn these parameters depend in a complex way on the external plasma parameters: reactor geometry, type of excitation, applied power, type of process gas, gas pressure and gas flow rate.

In many applications involving contact between polar liquids 20 and surfaces it is of no big concern whether an introduced hydrophilicity is stable towards washing or not. Particular problems are encountered in case the polymer surface is part of a channel of capillary dimensions, where a high degree of hydrophilicity is necessary if aqueous liquids are to be 25 introduced by self-suction or by centripetal forces. This becomes particularly true in case a repeated contact is to take place reproducibly, in which case an unstable surface modification will be washed away during the first liquid contact. The smaller dimensions of the channel the more severe 30 the problem becomes.

In the context of the invention the expression "plasma treated surface" will, if not otherwise specified, refer to an uncoated naked plasma treated surface, possibly being derivatized to contain separate reactive species firmly bound 35 to the surface.

Cell culturing in microfabricated devices has been described previously in for instance GB patent application 9808836.2 filed April 27, 1998. Microfluidic devices in which liquid transportation systems are defined by hydrophilic/hydrophobic

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barriers have been described previously in for instance GB patent application 9809943.5 filed May 8, 1998.

Objectives of the invention

- 5 • A first objective is to provide a gas plasma method for hydrophilisation of polymer surfaces, which enhances the stability of the hydrophilicity introduced.
- A second objective is to provide plasma treated surfaces that are hydrophilic after the treatment and
10 remain so upon repeated wetting/drying, i.e. have a initial hydrophilicity that is not significantly altered in contact with hydrophilic liquids, for instance etanol/water mixtures.
- A third objective is to provide capillary/channel/
15 chamber system, for instance in microfabricated form, having capillaries/channels/chamber, the inner surfaces of which being as defined for the second objective and permitting repeated introduction of aqueous solutions in a reproducible way.
- 20 • A fourth objective is to provide liquid transportation systems in which at least a part of the inner surfaces complies with the second objective.
- A fifth objective is to provide plasma treated surfaces
25 that can be used for cell culturing, assay reactions etc.

These objectives are mainly adapted to surfaces that before the plasma treatment have a relatively high immediate water-contact angle, for instance $\geq 20^\circ$, such as $\geq 30^\circ$ or even $\geq 50^\circ$.

By the term "immediate water-contact angle" is meant that the
30 contact angle is measured on a dry surface before an applied liquid has significantly evaporated. See the experimental part.

The invention

We have now discovered that by contacting a polymer surface
35 that is to be hydrophilised with a gas plasma having a high intensity (energy input per gas molecule) the objectives given above can be complied with. Our discovery is explainable in terms of two different types of introduced polar groups: Groups

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retained firmly on the polymer surface and groups allocated to loosely held degradation fragments. In the former case the groups will cause a stable hydrophilicity. In the latter case the hydrophilicity will easily be removed by contact with polar liquids, such as aqueous solutions.

Accordingly a first aspect of the invention is a method for rendering a polymer surface permanently more hydrophilic by contacting the surface with a gas plasma so that firmly bound polar groups are introduced on the surface. Most likely these polar groups are introduced directly on the polymer skeleton constituting the surface, possibly involving cross-linking of the surface layer.

Hydroxy and or amino groups, carboxy groups, ether groups etc and other groups in which a carbon atom binds to a heteroatom selected among oxygen, sulphur, and nitrogen are examples of polar groups that may be introduced. Changes in surface presence of this type of groups may be studied by ESCA (XPS).

The expressions "permanently more hydrophilic" and "stable hydrophilicity" contemplate that the immediate water-contact angle remains essentially unchanged upon washing with ethanol (70 % w/w, washing procedure as given in the experimental part). This means that that the washing procedure should not be allowed to change the immediate water-contact angle more than $\pm 20^\circ$ and/or more than $\pm 5^\circ$.

The storage stability (in dry form) of the hydrophilised surface should be at least one month with acceptable increases in immediate water-contact water angle not being larger than 10° , preferably not larger than 5° . In case the storage stability in dry form is not acceptable, sufficient storage stability should be accomplished by storing in aqueous atmosphere or in an aqueous liquid.

The method of the invention may have an optional washing step subsequent to the gas plasma treatment step. This washing procedure means contacting the gas plasma treated surface with an aqueous solution or some other polar liquid to remove loosely held hydrophilic compounds. The washing solution is preferably water, a water-miscible liquid or a mixture of

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these. Among water-miscible liquids may be mentioned methanol, ethanol, isopropanol, n-propanol, t-butanol, sec-butanol, dimethyl formamide, dimethyl sulphoxide, acetone and other liquid compounds having similar solubilities in water.

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The required intensity of the plasma will depend on the variables discussed above. Satisfactory gas plasmas may be found in case the electric excitation power applied is ≥ 250 W with preference for ≥ 300 W, and typically 500 ± 100 W with a gas flow selected in the interval of ≤ 50 cm³/min, with preference for ≤ 25 cm³/min. For the plasma intensity, the interval is normally ≥ 5 W/cm³/min, such as ≥ 10 W/cm³/min or ≥ 20 W/cm³/min or even ≥ 35 W/cm³/min. Normalised values per m² electrode area will typically be ≥ 30 W/m²/cm³/min, such as ≥ 60 W/m²/cm³/min or ≥ 120 W/m²/cm³/min or even ≥ 215 W/m²/cm³/min. Resulting pressure levels will be lower than 100 mTorr, with preference for pressures that are ≤ 50 mTorr. These ranges apply for a temperature of 25°C, atmospheric pressure and oxygen. For other gases the values must be multiplied with M_{O_2}/M_x , where M_{O_2} and M_x are the molecular weights of oxygen and the other gas, respectively.

The gases used should be non-polymerisable in the type of plasma contemplated. Typical such gases are inorganic. This means that suitable gases are found among oxygen, nitrogen, noble gases (such as helium, neon, argon, krypton, xenon) and mixtures thereof, such as air and mixtures containing other proportions of oxygen and nitrogen. Other potentially useful gases are carbon dioxide, carbon monoxide, water vapour etc that might be used either solely or in combination. By varying the composition of gas the groups inserted onto the surface it is likely that the groups can be varied with respect to kinds and density.

Illustrative examples of polymerisable gases are volatile lower organic molecules such as lower hydrocarbons and vapours of allyl- or acryl monomers, aromatics etc.

Plasma reactor vessels enabling a sufficiently high power output combined with proper gas flow velocities are

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commercially available. As stated above the reactor vessels should enable an electric excitation power input for instance in the microwave or radiowave ranges. A suitable plasma reactor is PS0500 (BOC Coating Technology, USA) which permits a
5 radiofrequency (RF) power of 0-500 W and gas flow of 0-100 or 0-1000 standard cm³/min.

The results of a treatment may depend on the design of the reactor vessel used meaning that the optimal interval to a certain degree will vary from one reactor design to another.
10 The results may also depend on where in the reactor the surface is placed during the treatment.

The immediate water-contact angle of the polymer material (plastics) covering the surface to be gas plasma treated is
15 typically $\geq 20^\circ$. The largest advantages with the invention are obtained for polymer materials that provide larger immediate water-contact angles, such as $\geq 30^\circ$ for instance $\geq 50^\circ$. These figures refer to plastic surfaces having been cleaned with respect to water-soluble compounds and low molecular weight
20 compounds (typically ≤ 1 kD). Illustrative examples of how the water-contact angle may vary with polymer are given in table 1. The inventive method typically should result in a decrease in the permanent hydrophilicity which corresponds to a lowering of more than 20 % such as more than 50 % of the immediate water
25 contact angle of the initial plastics surface (after removal of water-soluble compounds and the like). A higher decrease will be more important for hydrophobic than for hydrophilic plastics.

30 TABLE 1.

Plastic	Water-contact angle
PTFE (Teflon)	108°
Silicone rubber	
(Sylgard 184)	106°
35 Polypropylene	95°
Polyethylene	94°
Polystyrene	90°

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Polycarbonate 78°

PET (polyester) 76°

Styrene-acrylonitrile 73°

PMMA (Plexiglas) 59°

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Typically the polymer on the surface may be based on polymerisation of monomers comprising unsaturation such as in carbon-carbon double bounds or carbon-carbon-triple bonds/polymer/copolymer.

- 10 The monomers may, for instance, be selected from mono-, di and poly/oligo-unsaturated compounds, e.g. vinyl compounds and other compounds containing unsaturation. Illustrative monomers are:

- (i) alkenes/alkadienes (such as ethylene, butadiene, propylene and including substituted forms such as vinyl ethers), cycloalkenes, polyfluorovinyl hydrocarbons (for instance tetrafluoroethylene), alkene-containing acids, esters, amides, nitriles etc for instance various methacryl/acryl compounds; and
- 15 (ii) vinyl aryl compounds (such as mono-, di- and trivinyl benzenes) that optionally may be substituted with for instance lower alkyl groups (C1-6) etc.
- 20

Another type of polymers are condensation polymers in which the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc groups. Particularly emphasised monomers are polyamino monomers, polycarboxy monomers (including corresponding reactive halides, esters and anhydrides), poly hydroxy monomers, amino-carboxy monomers, amino-hydroxy monomers and hydroxy-carboxy monomers, in which poly stands for two, three or more functional groups. Polyfunctional compounds include compounds having a functional group that is reactive twice, for instance carbonic acid or formaldehyde. The polymers contemplated are typically polycarbonates, polyamides, polyamines, polyethers etc. Polyethers include the corresponding silicon analogues, such as silicone rubber.

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The polymers may be in cross-linked form.

The polymer on the surface may be a mixture of two or more different polymer(s)/copolymer(s). In the latter case the water-contact angles discussed above apply to these mixture, i.e. the factual surface.

Particularly interesting polymers are those that have a non-significant fluorescence for excitation wavelengths in the interval 200-800 nm and emission wavelengths in the interval 400-900 nm. By non-significant fluorescence is meant that the fluorescence intensity in the above-given emission wavelength interval should be below 50 % of the fluorescence intensity for a reference plastics (= a polycarbonate of bisphenol A without fluorescent additives). In fact it does not harm in case the fluorescence intensity of the plastics is even lower, such as < 30 % or < 15 %, such as < 5 % or < 1 %, of the fluorescence intensity of the reference plastics. Typical plastics having an acceptable fluorescence are polymers of aliphatic monomers containing polymerizable carbon-carbon double bonds, such as polymers of cykloalkenes (e.g. norbornene och substituterade norbornenes), ethylene, propylenes etc, as well as other non-aromatic polymers of high purity, e.g. certain grades of polymethylmethacrylate.

The requirement for a low fluorescence is of particular importance in case the plastics are to be used for carrying samples in which fluorescent substances are to be detected/measured. It then becomes important to select plastics with non-significant fluorescence at the wavelength at which the substance to be detected/measured fluoresces. In most cases this means that the fluorescence intensity of the plastics at the emission wavelength of the substance should be below 50 % of the fluorescence intensity of the substance. In fact it will not harm in case the fluorescence intensity of the plastics is even < 30 %, such as < 15 %, or still lower, such as < 5 % or < 1 %, of the fluorescence intensity of the substance. In case the fluorescence of several substances emitting light at different wave-lengths are to be measured it will put harsher demands on the plastics, since fluorescence of the plastics

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then should be non-significant for broader wave-length bands or for several bands.

After the plasma treatment, optionally after a washing
5 procedure to remove loosely held hydrophilic compounds, the surface may be further derivatized to exhibit one or more type of reactive groups, i.e. groups that are able to bind other compounds either via some type of affinity or via covalent linking. Derivatization preferably takes place after the
10 washing step and may be preceded by a coating step, for instance, to provide the surface with a coating carrying additional hydrophilic groups that can be used for derivatization.

15 By masking certain parts of the surface and leaving other parts unmasked before hydrophilisation, hydrophilic patterns on a hydrophobic surface can be obtained. Alternatively a hydrophobic pattern may be printed on the surface after the hydrophilisation. These techniques may be of value in the
20 manufacture of microfabricated liquid transportation systems. See below.

Various methods for introducing reactive groups on polymers exhibiting hydrophilic groups, such as hydroxy, amino or
25 carboxy etc groups are well known to the averaged skilled artisan in the field. Well known affinity groups are charged groups, and groups exerting affinity via interactions of other types, possibly in combination with charge-charge interactions. Illustrative examples of charged groups are ion-exchanging
30 groups, such as anion and cation exchanging groups, with typical examples being ammonium ions (primary, secondary, tertiary and quaternary ammonium ions), sulphates, sulphonates, phosphates, phosphonates etc. Illustrative examples of other affinity groups are so called bioaffinity groups including
35 individual members of ligand - receptor pairs, such as antibody - antigen/hapten, complementary nucleic acids, Ig binding proteins - Ig (e.g. protein A or G - IgG), lectins -- carbohydrate structures, cells - cell attachment molecules (fibronectin, collagen, RGD-peptides) etc. Included in

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bioaffinity groups are also semi- and fully synthetic ligands that more or less completely mimics native bioaffinity.

The hydrophilised surface may be part of arrangements of 5 different physical forms. The hydrophilised surface may be the bottoms/walls of microtiter wells and other types of vessels and also the outer surfaces of porous and non-porous particle material. The hydrophilised surface may be part of a number of surfaces on a less hydrophilic larger surface (e.g. 10 hydrophobic).

The hydrophilised surface may provide a significant part of the liquid contact surfaces in systems intended for transport of aqueous liquids. The type of systems may have channels that may be of capillary dimensions, for instance with 15 a distance between two opposite walls being $\leq 1000 \mu\text{m}$, such as $\leq 100 \mu\text{m}$, or even $\leq 10 \mu\text{m}$, such as $\leq 1 \mu\text{m}$. This type of systems may also contain one or more chambers connected to the channels and having volumes being $\leq 500 \mu\text{l}$, such as $\leq 100 \mu\text{l}$ and even $\leq 10 \mu\text{l}$ such as $\leq 1 \mu\text{l}$. The depths of the chambers may 20 typically be in the interval $\leq 1000 \mu\text{m}$ such as $\leq 100 \mu\text{m}$ such as $\leq 10 \mu\text{m}$ or even $\leq 1 \mu\text{m}$.

One or more liquid transportation systems of this type may be placed on a common plate, for instance rotatable, such as a disc of CD-type. In case of rotatable forms the liquid may be 25 forced through one or more segments of the transportation system by rotating the disc (centripetal force). Other types of pressure generating systems may also be used.

A device having one or more liquid transportation system comprising channels and chambers with a depth $\leq 1000 \mu\text{m}$, such 30 as $\leq 100 \mu\text{m}$ or even grounder than 10 such as $\leq 1 \mu\text{m}$, are further on called a microfabricated device. The chambers/channels are said to be in the microformat. A microfabricated device typically has its channels and chambers in one plane, such as in the surface of a plate, for instance 35 on a disc. The plate may be circular, oval, rectangular (including in form of a square) or of any other 2D geometric form.

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The channels and/or chambers are defined by liquid barriers guiding a liquid flow. The liquid barriers can be in form of physical walls, bottoms and tops. Hydrophobic barriers combined with aqueous liquids and vice versa for non-polar liquids (see GB patent application 9808836.2 filed April 27, 1998) has been suggested for directing a liquid flow, e.g. to replace a wall and the like. This is likely to be important in order to define channels/chambers in liquid transportation systems in microfabricated devices. By covering a surface (I) to be hydrophilised with a mask leaving a pattern of communicating lines and dots unmasked and hydrophilise, for instance according to the present invention the surface (I) will exhibit a hydrophilic pattern. When placing a purely hydrophobic surface (II) against the hydrophilic pattern leaving a capillary slot between the surfaces, a liquid transportation system will be obtained. Surface (II) may also have a hydrophilic pattern matching the hydrophilic pattern of surface (I). As discussed above the hydrophilic pattern may also be obtained by hydrophilise the full surface and then print the desired hydrophobic pattern thereon.

In microfabricated devices having the pattern of a liquid transportation system defined on a surface as described in the preceding paragraph, there is also a second surface applied against the pattern and acting as a top covering the pattern and preventing evaporation of liquid (except for minor parts/dots intended for addition/removal of liquids).

Liquid transportation systems of the type referred to above may also contain valves, pumps, filters and the like.

The surface may be used for performing chemical reactions of inorganic and/or organic/biochemical nature. The surface may be used as carrier matrix in chromatography, for cell culture, for solid phase chemical synthesis of oligo/polypeptides, oligo/polynucleotides, other organic polymers and other organic compounds. Illustrative examples of reactions to be run on the surface of the invention are conventional chemical reactions or reactions that are based on affinity involving recognition through geometric fit and interactions based on hydrogen-bonding, van-der Waals bonding, dipole-dipole interaction,

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charge-dipole interaction, charge-charge interaction etc.

Vessels having interior surfaces being treated according to the invention may be used for storage of various types of organic and inorganic chemicals and/or liquids. For cell culturing, 5 further details are given below.

A second aspect of the invention is a naked plasma treated polymer surface permanently hydrophilised as defined above and complying with anyone of the other above-mentioned features, 10 either alone or in combination.

A third aspect of the invention is the various uses discussed above or below for surfaces treated in accordance with the invention and/or having any of the features discussed herein 15 and achievable through the inventive hydrophilisation method.

Kit containing a microfabricated device

A fourth aspect is a kit containing

(a) a microfabricated device comprising a surface (i) 20 which (i) is manufactured from a synthetic polymer material (plastics) and (ii) on which there are at least one chamber and/or at least one channel, and

(b) a fluorescent substance to be detected in the device, The kit is characterized in that the synthetic polymer material 25 has a fluorescence that is non-significant in the same sense as discussed previously in this specification. The surfaces in the channels and chambers of the microfabricated device may wholly or partly be hydrophilised, for instance by gas plasma treatment, preferably according to the method described herein.

30 With respect to the chemical composition of the plastics of the surface and its physical parameters, the same material with the same preferences as given previously may be used. See above.

Alternative hydrophilisation protocols are treatment with oxidating acids and with UV-oxidations, corona treatment, 35 grafting and conventional coating with a polymer providing an increased number of polar groups, etc on the liquid contact surface of the material. The polar groups referred to are for instance hydroxy, amino, carboxy, amido, polyethylene oxide etc.

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Illustrative examples of fluorescent substances are fluorophores of organic or inorganic origin. In the former case they often have a low molecular weight (typically < 1 kD). Important fluorophors have distinct emission wavelengths with distinct maxima in the interval 400-750 nm, with preference for the interval 480-670 nm. Fluorescein, phycocyanines that may be native or chemically modified, rhodamine, Texas Red, fluorescent rare earth chelates (in particular europium and terbium), cadmium selenide nanoparticles etc are typical examples. The kit, in particular the chambers and the channels combined with the fluorescent substance, may be used for running chemical reactions, assays, separations, culturing cells and the like as described elsewhere in this in this specification.

15

A microfabricated device.

A fifth inventive aspect is a microfabricated device as defined above, in which the liquid transportation system is formed in/on a polymer material (plastics) in which the plastics essentially consist of one or more polymers obtained by polymerising one or more aliphatic monomers of the kind defined above. The device may have one or more of the features of the other inventive aspects described in this specification, except for the unique selection of polymer material. The same uses can be applied.

Cell culturing

A sixth inventive aspect is a method for culturing cells.

Culturing of cells, in particular anchorage-dependent cells, have previously been carried out in the presence of substrate surfaces made of plastics. The immediate water-contact angle has been 40° - 60° . For microfabricated devices, i.e. devices having chambers/channels with this relatively low hydrophilicity, there will be problems with respect to liquid fluidics.

It has now been found that culturing of various kinds of cells can be carried out in contact with superhydrophilic substrate surfaces made of plastics having an immediate water-

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contact angle that is significantly lower than 40°-60°. When applied to microfabricated devices this discovery will improve the situation with respect to liquid fluidics. This aspect thus is characterized in that the cell culturing takes places in the presence of a surface made of plastics providing an immediate water-contact angle $\leq 35^\circ$, such as $\leq 30^\circ$ or even below 20°. The surfaces are primarily provided by the walls of the culture vessel, but may also be provided by e.g. particles suspended therein.

10 The immediate water-contact angle refers to hydrophilicities that are stable against repeated washing as described above.

Surfaces that have been hydrophilised by gas plasma treatment as described in this specification are preferred. The preferred plasmas contain one or more gases that solely or in combination can introduce the mix of charged/polar groups discussed below. Among the gases tested, mixtures of oxygen and nitrogen are most preferred, with the individual gases being less preferred and with argon being least preferred. One can envisage that gases, such as sulphur dioxide and diphosphorous pentoxide, might be beneficial to use together with oxygen and/or nitrogen, if disregarding the handling problems they might give.

Alternative hydrophilisation protocols are given in the context of the fourth inventive aspect.

25 The most important factor for successful culture and behaviour of anchorage-dependent cells is the surface on which the cells grow. If this type of cells are plated onto a surface to which they cannot adhere they will not grow. Cell function require dynamic interactions between the cell and its substratum. These interactions occur at specialised contact sites where transmembrane proteins (integrins; Hynes RO, Integrins: versatility, modulation and signaling in cell adhesion. Cell 69: 11-25, 1992) link the interior of the cell with the external substratum. With respect to a material surface, surface functional groups and their charge character as well as hydrophilicity/hydrophobicity and surface free energy appear important for cell behaviour (Lee JH et al., Biomaterials 18:351-358. 1997) A variety of polar and charged functional groups such as amine (ammonium), amide, hydroxyl,

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carboxyl (carboxylate) and sulphonyl (sulphonate) and sulphate groups at a suitable density are considered as sites for electrostatic interaction with the cell surface or attachment proteins and as mimicking adhesive contacts on extracellular matrix components (Maroudas, J. Theor. Biol. 49 (1975) 417-442; Lee et al., Biomaterials 15 (1994) 704-711; and Lee et al., Biomaterials 18 (1997) 351-358).

Non-anchorage dependent cells often require substrate surfaces during a certain part of their life cycle.

Typically the density of charged groups should be above 1-2 group per \AA^2 . As a thumb of rule smaller cells, such as HeLa cells (10-20 μm), require higher densities than larger cells, such as fibroblasts (about 30x100 μm), lower densities. The optimal values for a certain kind of cell vary among cell types and may be determined as known in the art.

Cell culturing according to this aspect of the invention applies to a wide variety of cells. The cells may be anchorage- or non-anchorage-dependent. They may be of normal or tumour origin and they may be genetically manipulated in culture. They may be derived from mammals, bacteria, fungi (yeast), plants, fish, birds, amphibians, reptiles, etc. With respect to mammalian cells they may derive from any tissue, e.g. epithelial, endothelial, fibroblast, muscle, nerve, pigment, hematopoietic and germ cells.

For each respective kind of cell, the rules for selecting conditions and protocols are in principle the same as for culturing in other vessels and on particles.

The polymer surface material should not be toxic to the cells to be cultured. We have, for instance recognized, that gas plasma treated polymers built up of acrylo nitrile monomer or acrylate monomer may be toxic, probably due to degradation of the polymer. This may be circumvented by avoiding these kinds of material or by a proper post treatment of the material before it is used.

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The invention will now be illustrated by non-limiting experiments. The invention is further defined in the appended claims that are part of the application text.

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EXPERIMENTAL PART

Materials

5 Surfaces (discs): Polycarbonate of bisphenol A and polymethylmethacrylate CD blanks, injection molded at Toolex Alpha AB, Sundbyberg, Sweden. Non-patterned CD blanks, injection molded from Zeonex (a cycloolefin copolymer from Nippon Zeon, Japan) or Luran KR2536 (a styrene-acrylonitrile 10 copolymer (SAN) from BASF, Germany) at Åmic AB, Uppsala, Sweden). The planar (non-patterned) side of the discs were used in all experiments.

Gases: Oxygen, Argon and synthetic air were from l'Air Liquide, France.

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Plasma reactor: Plasma Science, PS0500 (BOC Coating Technology) main adjustable parameters: Radiofrequency (RF) power 0-500 W and gas flow 0-100 or 0-1000 sccm (standard cm³/min)

20 Normally the reactor PS0500 is equipped with three electrode plates but after a rebuilding only one plate remained.

Methods

Washing: Before plasma treatment all discs were immersed in pro 25 analysi isopropanol for 2 min, briefly flushed with 99% ethanol and blown dry with house nitrogen. This was done to remove any release agents, antistatic agents etc which might interfere with the plasma treatments.

30 Plasma treatments: The discs were placed in the plasma reactor in one of two positions; either on a plastic support 20.5 cm from the chamber floor or on glass supports placed on the electrode plate (45 cm from the chamber floor). After evacuation to a base pressure of 60 mTorr, the gas was let in 35 and the gas flow adjusted to the desired level. The RF power was then switched on for the intended time and the reactor chamber was finally vented with ambient air.

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Contact angle measurements: Directly after treatment, the equilibrium water-contact angle was measured with the sessile drop method on a Ramé-Hart goniometer bench. For each sample 5 six measurements were made (two sides on each of three droplets). Contact angle measurements were also made after the pieces had been immersed for 2 min in 70% ethanol/water and blown dry with house nitrogen. The measurement was made within 10 20 s after the liquid had been applied in order to avoid changes in contact angle e.g. due to evaporation of the droplets.

Check for introduction of cross-links: The polymer material was dissolved in a suitable solvent for original polymer but 15 not for polymer chains cross-linked during the plasma treatment before and after gas plasma treatments. The presence of any insoluble material after treatment was taken as an indication of introduction of cross-links.

20 Check for introduction of polar oxygen containing groups:

Preliminary studies by ESCA showed that the pattern of these groups in the surface changed upon gas plasma treatment in a way suggesting an increase in surface bound oxygen. ESCA can be used to determine the various polar/charged groups that may be 25 of importance for cell culturing.

Storage study: The plasma treated discs were placed in polystyrene Petri dishes and stored under ambient lab conditions. With regular intervals small pieces were cut off 30 and the immediate water-contact angle measured both directly and after immersion in 70% ethanol/water. The cut-off samples were discarded after measurement.

Criteria for acceptance: From the hydrophilicity point of view 35 the preliminary acceptance criterion was that the water-contact angle should be 20° or lower after washing in 70% ethanol.

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Cell culture:**Methods for evaluating surfaces:**

Cell culture was used as a method for evaluation of the plasma-treated surfaces, since cells pose extremely high demands on their substratum.

Pieces of plasma-treated material were placed in multi-well plates, a suspension of cells in culture medium was added to each well and the plates were incubated in a cell culture atmosphere for various times. Cell adhesion, morphology and proliferation were evaluated microscopically and occasionally by the use of immunocytochemistry against cell proliferation markers and adhesive contacts. Preliminary results have revealed that the plasma-treated surfaces can be used for cell culture and that the cells exhibit necessary characteristics for optimal behaviour. Such characteristics include adhesion of nearly all of the plated cells in a evenly pattern, proper cell spreading on the material surface, signs of normal cell motility and cell division. Pathological signs including vacoles, excessive amounts of lysosomal granulae, blebbing or membrane destruction were insignificant. The cell lines tested so far include MRC5 (normal lung fibroblasts), HeLa (cervix carcinoma cells of epithelial-like origin), Chang (hepatoma, liver cells). The cells have been selected so that they will cover a wide range of demands on the surfaces. Non-anchorage dependent cells put very small demands on the surfaces as such. An example of such cells (Raji lymphoma), have been successfully cultured in the presence of the plasma-treated surfaces.

RESULTS**Planar CD discs plasma-treated**

Plasma Science PS0500 reactor with one electrode plate

Samples placed on a polypropylene support 20 cm from the reactor chamber floor in the center of the chamber

Disc material	Gas	Gas flow set value	RF power	Power/flo w	Plasma time	Contact angle direct	Contact angle washed in
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PMMA	air (synth)	10	300	30	5	6	14
PMMA	air (synth)	5	500	100	5	8	4
PMMA	air (synth)	5	300	60	5	5	3
PMMA	oxygen	50	500	10	5	29	54
PMMA	oxygen	50	300	6	5	39	52
PMMA	oxygen	25	500	20	5	11	40
PMMA	oxygen	25	300	12	5	31	53
PMMA	oxygen	10	500	50	5	5	10
PMMA	oxygen	10	300	30	5	7	45
PMMA	oxygen	5	500	100	5	4	4
PMMA	oxygen	5	300	60	5	4	7

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C L A I M S

1. A method for rendering a surface covered by a polymer material (plastics) more hydrophilic by treatment in a gas plasma of a non-polymerizable gas, characterized in that the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic, and with the optional step that the surface subsequently is washed with a solvent selected from water, a water-miscible solvent and mixtures thereof.
2. The method according to claim 1, characterized in that the plasma intensity is $\geq 5 \text{ W/cm}^3/\text{min}$, in particular with a power $\geq 250 \text{ W}$ and a flow $\leq 50 \text{ cm}^3/\text{min}$.
3. The method of any one of claims 1-2, characterized in that
 - (a) the polymer material has been selected among plastics having an immediate water-contact angle $\geq 20^\circ$ and
 - (b) the plasma treatment conditions are set so that the immediate water-contact angle after plasma treatment and a subsequent wash with ethanol (70 % w/w) becomes $\leq 30^\circ$, such as $\leq 20^\circ$.
4. The method of any one of claims 1-3, characterized in that
 - (a) polymers/copolymers in which the monomers are unsaturated such as (i) alkenes/alkadienes (such as ethen, propen, butadiene and including substituted forms such as vinyl ethers, polyfluorovinyl hydrocarbons (for instance tetrafluoroethylene), and acids, esters, amides, nitriles etc containing one or more alkene groups, for instance various methacryl/acryl compounds; (ii) vinyl aryl compounds in which the vinyl group is bound to aryl (such as mono-, di- and trivinyl benzenes) that optionally may be substituted with for instance lower alkyl groups (C1-6); etc;
 - (b) condensation polymers/copolymers in which the monomers are selected from compounds exhibiting two or more

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groups selected among amino, hydroxy, carboxy etc groups.

5. The method of any one of claims 1-4, characterized in that
5 the plasma is induced by radio- and/or microwaves.

6. The method of any one of claims 1-5, characterized in that the plasma gas is oxygen, nitrogen, or a noble gas, such as argon, or a mixture of these gases.

10

7. The method of any one of claims 1-6, characterized in that subsequent to the plasma treatment the surface is derivatized to exhibit positively, negatively and amphoteric groups; hydroxy groups, bioaffinity groups, chelating groups etc.

8. The method of any one of claims 1-7, characterized in that at least part of the surface is fabricated into a liquid transportation system of a microfabricated device.

20

9. A naked plasma treated surface of plastics characterized in that the surface has an immediate water-contact angle $\leq 30^\circ$, such as $\leq 20^\circ$, said water-contact angle being changed less than $\pm 20\%$ and/or less than $\pm 5^\circ$ upon washing with ethanol/water mixture (70% w/w).

10. The naked surface of claim 9, characterized in that the surface is made up of a polymer material selected from

(a) polymers/copolymers in which the monomers are unsaturated such as (i) alkenes/alkadienes (such as ethen, propen, butadiene and including substituted forms such as vinyl ethers, polyfluorovinyl hydrocarbons (for instance tetrafluoroethylene), and acids, esters, amides, nitriles etc containing one or more alkene groups, for instance various methacryl/acryl compounds; (ii) vinyl aryl compounds (such as mono-, di- and trivinyl benzenes) that optionally may be substituted with for instance lower alkyl groups (C1-6); etc;

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- (b) condensation polymers/copolymers in which the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc groups;
- 5 said polymer material optionally being cross-linked and/or optionally being a mixture of two or more kinds of polymers/copolymers.
11. The naked surface of any one of claims 9-10, characterized
10 in that the polymer material before having been gas plasma treated exhibits an immediate water-contact angle $\geq 30^\circ$.
12. The naked surface of any one of claims 9-11, characterized
15 in that at least part of the naked surface is part of a liquid transportation system of a microfabricated device.
13. The use of the surface defined in any one of claims 9-11 and/or obtained by the method defined in any one of claims 1-8 for culturing cells.
- 20 14. A kit comprising
- (a) a microfabricated device comprising a surface (i) which is manufactured from a synthetic polymer material (plastics) and (ii) on which there are at least one
25 chamber and/or at least one channel, and
- (b) a fluorescent substance to be detected in the device, characterized in that the plastics has a fluorescence that is non-significant with respect to the fluorescence, in particular with the fluorescence intensity of the plastics
30 being $< 50\%$ of the fluorescent intensity of the substance at the wavelength at which the substance fluoresces.
15. A method for culturing cells that in at least some part of their life cycle require attachment to a substrate surface,
35 characterized in that the substrate surface provides a plastic surface having an immediate water-contact angle $\leq 30^\circ$.

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16. The method of claim 15, **characterized** in that the plastic surface having an immediate water-contact angle $\leq 30^\circ$ has been obtained by the gas plasma treatment method defined in claims 1-8 of a surface made of plastics.

5

17. The method of any one of claims 15-16, **characterized** in that the cell culturing is carried out in a chamber of a microfabricated device.

10 18. A microfabricated device in which the liquid transportation system is formed in/on a polymer material (plastics) that comprises a polymerised aliphatic monomer containing unsaturation.

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A B S T R A C T O F T H E D I S C L O S U R E

A method for rendering a surface covered by a polymer material (plastics) more hydrophilic by treatment in a gas plasma of a non-polymerizable gas. The method is characterized in that the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic.

A naked plasma treated surface of plastics having an immediate water-contact angle $\leq 30^\circ$, such as $\leq 20^\circ$, said water-contact angle being changed less than $\pm 20\%$ and/or less than $\pm 5^\circ$ upon washing with ethanol/water mixture (70% w/w).

A kit comprising (a) a microfabricated device comprising a surface (i) which is manufactured from a synthetic polymer material (plastics) and (ii) on which there are at least one chamber and/or at least one channel, and (b) a fluorescent substance to be detected in the device. The kit is characterized in that the plastics has a fluorescence that is non-significant with respect to the fluorescence of the substance at the wavelength at which the substance fluoresces.

A microfabricated device having a liquid transportation system which is formed in/on a polymer material (plastics) that is a polymerised aliphatic monomer containing unsaturation.